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Augmentation of Leukocyte Infiltration in Murine Tumors Expressing B-Cell Derived but Not Nasopharyngeal Carcinoma Derived EBV Membrane Protein LMP1

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The Epstein-Barr virus (EBV) encoded latent membrane protein of B cell origin, B-LMP1 (B95-8 prototype) and nasopharyngeal carcinoma (NPC) derived C-LMP1 (CAO prototype) were transfected individually in S6C adenocarcinoma cells of ACA (H-2f) origin. We have shown previously that inoculation of B-LMP1 expressing S6C cells led to tumor rejection in pre-immunized, immunocompetent syngeneic ACA mice, whereas the C-LMP1 transfectants were not immunogenic. Furthermore, B-LMP1 but not C-LMP1 expressing S6C cells grew with necrosis and extensive skin damage in non-immunized mice. A study was carried out to determine whether the in vivo growth pattern of S6C cells expressing two different LMP1 isolates could be correlated to any immunomodulatory mechanism. An increased infiltration of CD45+ leukocytes was found in B-LMP1 expressing S6C tumors originating in non-immunized, syngeneic ACA mice. The C-LMP1 expressors, vector transfectants and untransfected parental tumors had significantly lower number of infiltrating leukocytes. The immunoaccessory molecules ICAM-1, B7-1 and MHC Class I and II expression was unaltered in both B- and C-LMP1 transfectants. The data suggest that B-LMP1 but not C-LMP1 induce anti-tumor immune response. ***J. Med. Virol. 60:417–424, 2000.***

KEY WORDS: EBV; LMPI; NPC; BL; leukocytes

INTRODUCTION

EBV is unique among herpesviruses in its ability to transform B cells. The resulting lymphoblastoid cell lines (LCLs) express nine EBV encoded growth transformation associated proteins EBNA1-6, LMP1, 2A and 2B [Kieff and Leibowitz, 1989]. The LCLs are strong stimulators of both autologous and allogeneic T cell responses. The induction of the T cell responses by LCLs can partly be attributed to its immunoblastic phenotype. In contrast, phenotypically representative Burkitt lymphoma (BL) lines are poor stimulators of T cells [Avila-Carino et al., 1987]. This could be due to the lack of potentially immunogenic EBV encoded proteins, low expression of adhesion molecules and certain HLA elements [Gregory et al., 1988; Masucci et al., 1995].

Once infected, an individual carries EBV for life. The outgrowth of spontaneous LCLs from explanted lymphocytes of healthy carriers is inhibited in vitro. The cytotoxic T lymphocytes (CTLs) generated in such mixed autologous cultures lyse EBV transformed LCLs in a specific MHC restriction [Wallace et al., 1985]. In contrast, individuals with congenital or acquired immunodeficiencies are prone to EBV associated lymphoproliferations. These observations confirm the notion that EBV infected cells are tightly controlled by a functional immune system. More recent studies have identified that EBNA2-6 and LMPs, but not EBNA1 can serve as targets for such responses in appropriate MHC restrictions [Khanna et al., 1992; Gavioli et al., 1992; Murray et al., 1992; Lee et al., 1993].

The immunogenicity of another paradigmatic EBV carrying tumor, NPC is poorly understood. The NPC cells express EBNA1 but fail to express EBNA2-6. A majority of these tumors also express LMP1 and LMP2A [Fahraeus et al., 1988; Young et al., 1988; Chen et al., 1995].

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Murine model system has been used extensively to study the immunogenic potential of human papilloma virus genes [Chen et al., 1992]. Using such a system, it was demonstrated that although B-cell derived LMP1 isolate (B-LMP1) was highly immunogenic, its CAO NPC derived counterpart (C-LMP1) failed to induce rejection of the transfected S6C mammary carcinoma cells in the immunized, syngeneic ACA (H-2f) mice [Trivedi et al., 1994]. In the same study, it was also observed that in non-immunized ACA mice, the B-LMP1 transfected tumor grew with necrotic areas and heavy skin damage. In contrast, the C-LMP1 expressing S6C cells grew as well as nontransfected control S6C, without skin damage. The present study set out to investigate this difference in the growth of B-LMP1 and C-LMP1 expressing S6C in immunocompetent, non-immunized, syngeneic mice. An elevated number of tumor infiltrating leukocytes (TILs) was found in the B-LMP1 expressing S6C derived tumors as judged by CD45 reacting cells in frozen sections. The tumors carrying C-LMP1 or the vector alone and untransfected tumors had significantly lower number of CD45 positive leukocytes. Both B- and C-LMP1 carrying S6C express similar levels of ICAM-1, B7-1, MHC Class I and II, the immunoregulatory proteins required for efficient recognition by the CTLs.

MATERIALS AND METHODS

Cells

S6C is a spontaneous murine mammary adenocarcinoma line from ACA (H-2f) mouse [Kuzumaki et al., 1980]. The cells were maintained in RPMI supplemented with 10% fetal calf serum (FCS), 100 IU of penicillin and 100 μg/ml streptomycin. The B-LMP1 and C-LMP1 transfected S6C sublines are described elsewhere in detail [Trivedi et al., 1994].

Detection of LMP1 in S6C Cells

LMP1 was detected by immunoblotting. Briefly, 10^7 cells were lysed in 1 ml of the lysis buffer. Extracts from equal number of cells (10^6) were loaded in each lane. The proteins were separated by discontinuous gel electrophoresis and blotted onto nitrocellulose filters (Schleicher and Schuell, Dassel, Germany) [Towbin et al., 1979]. The transfer efficiency was verified by staining the filters with Ponceau S (Sigma). The filters were submerged in 5% dry skimmed milk made in phosphate buffered saline (PBS) for two hours to block excess protein binding sites. The filters were incubated later with anti-LMP1 monoclonal antibodies CS1-4 (Dako). After the incubation with the first antibody, the filters were washed 3 times in 5% milk in PBS and incubated with horseradish peroxidase conjugated anti-mouse antibodies. The LMP1 was visualized using the ECL kit (Amersham).

Immunohistochemical Analysis

The ACA (H-2f) mice were inoculated with 10^4 B-LMP1, C-LMP1, vector expressing and nontransfected S6C cells. After 6–8 weeks, the tumors were excised and were divided in parts that were either fixed in 10% buffered formalin, for subsequent paraffin sectioning and hematoxylin-eosin staining or were snap frozen for subsequent cryostat sectioning. The morphological identification of tumor cells, vessels and leukocytes was verified by immuno-peroxidase staining using monoclonal antibodies against smooth muscle actin (Dakopatts, Glostrup, Denmark) and antimouse CD45 (Boehringer Mannheim, Germany) respectively. A conventional three step indirect method was used with peroxidase-conjugated antisera (Dakopatts) in the second and the third step. Diaminobenzidine was used as chromogen (Sigma). Estimation of the number of immunostained cells per unit area in tumor sections was made by grid counting on videomicroscopic images. The number of immunostained cells "hitting" the grid was recorded as number of positive cells per unit area. A minimum of 25 high power fields were analyzed per tumor.

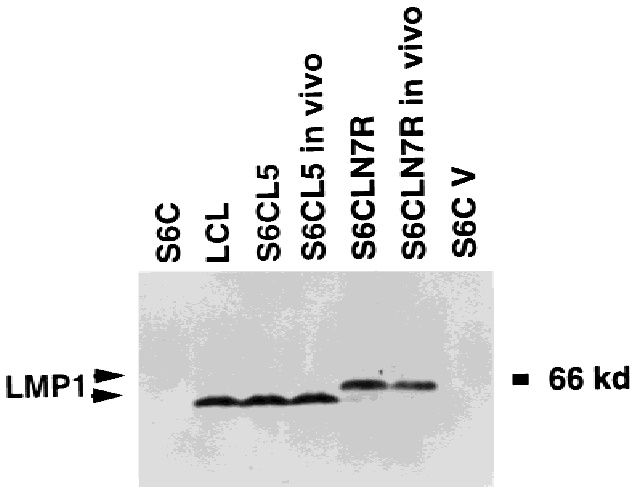

Fig. 1. LMP1 expression in S6C cells. Total cell extracts from the B-LMP1 and C-LMP1 expressing S6C clones were separated on a discontinuous 7.5% SDS-polyacrylamide gel. LMP1 was visualized by ECL system (Amersham) using CS1-4 monoclonal antibodies. Clone S6CL5 and S6CLN 7R express B- and C-LMP1 respectively. The tumors arising in syngeneic ACA mice (S6CL5 in vivo and S6CLN7R in vivo) were also examined for LMP1 expression. S6CV carry vector only.

Detection of ICAM-1 and B7-1 on LMP1 Expressing S6C Cells

FITC conjugated antibody to mouse CD54 (Pharmingen) was used to measure levels of ICAM-1 expression. The expression of B7-1 on B- and C-LMP1 expressing S6C was verified by using an antibody to mouse CD80 (Pharmingen). The percentage positive cells and fluorescence intensity was determined by FACS analysis.

RESULTS

LMP1 Expression in the Transfected S6C Cells and Tumors

As seen in Figure 1, the S6C B-LMP1 expressing clone S6CL5 and C-LMP1 carrying clone S6CLN7R ex-

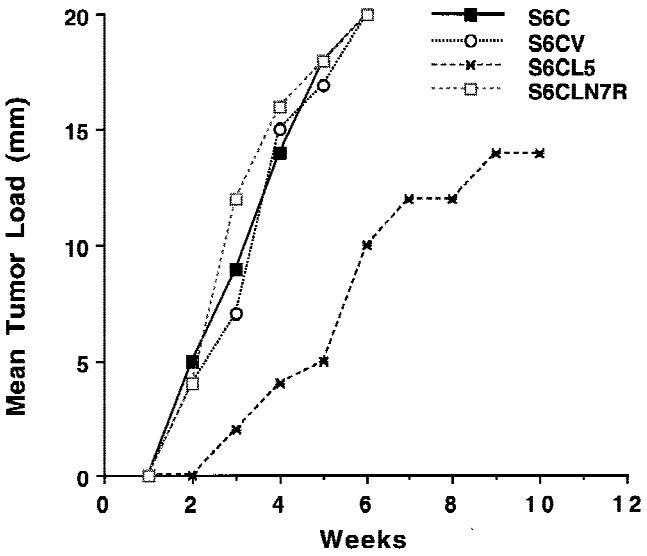

Fig. 2. Growth of untransfected, vector, B- and C-LMP1 transfected S6C in non-immunized, syngeneic, immunocompetent ACA mice; 10^4 cells were subcutaneously inoculated. The mean tumor load was calculated by adding the tumor diameters and dividing the sum by total number of mice inoculated.

TABLE I. Necrotic Growth of B- and C-LMP1 Expressing S6C Cells in Syngeneic Mice

		Tumor growth with necrosis/mice with tumor[a]	
Cells	LMP1-isolate	Syngeneic ACA	SCID
S6C	—	0/14	0/15
S6C V	—	0/12	0/13
S6CL5	B-LMP1	18/18	0/16
S6CLN7R	C-LMP1	0/20	0/18

[a]Mice were inoculated with 10^4 cells.

pressed high levels of LMP1. As an additional control, the B- and C-LMP1 expression in S6C transfectant clones was checked by immunofluorescence and 100% positivity was observed using CS1-4 antibodies (not shown). The expression of LMP1 was verified also in the tumors growing in syngeneic mice. The size of C-LMP1 was 66 kd compared to 63 kd of B-LMP1. This is due to 18 amino acids difference between the two LMP1 isolates as described previously by us [Hu et al., 1991; Trivedi et al., 1994].

In Vivo Tumor Formation

The S6C, S6CV, S6CL5 and S6CLN7R cells (10^4) were inoculated in non-immunized, immunocompetent, syngeneic ACA mice. Figure 2 shows that B-LMP1 expressing clone S6CL5 grew poorly compared to the C-LMP1 expressor S6CLN7R and vector alone carrying S6C in ACA mice. The tumors with extensive skin damage were observed only in B-LMP1 S6C inoculated ACA mice (Table I). When inoculated in SCID mice, both B- and C-LMP1 transfected S6C cells grew progressively without necrosis and skin damage (Table 1).

Parental, vector or B- and C-LMP1 expressing S6C cells formed invasive, poorly differentiated tumors in the non-immunized syngeneic ACA mice. The parental and the vector transfected S6C cells formed highly proliferative tumors with morphological appearance of a poorly differentiated carcinoma having pleomorphic, rounded to polygonal cell nuclei. In contrast, both B-LMP1 (S6CL5) and C-LMP1 (S6CLN7R) expressing cells formed tumors with more elongated cell nuclei (Fig. 3A,D). In the B-LMP1 derived tumors, the tumor cells were less densely packed, apparently surrounded by an increased amount of vascular stroma (Fig. 3C,F). In addition, the B-LMP1 expressing S6CL5 tumor cells were less proliferative and had condensed nuclear chromatin (Fig. 3A).

The C-LMP1 expressing S6CLN7R clone formed bundles of parallel tumor cells with a more sarcoma-like growth pattern. These tumors also had a high frequency of mitoses and larger cell nuclei with an open chromatin and multiple nucleoli. In addition, some of these tumors showed areas with extended sinusoidal venules forming hemangioma-like areas. In the case of B-LMP1 expressing S6CL5 derived tumors, some of the degenerative foci were bordered by aggregations of leukocytes.

Tumor Infiltrating Leukocytes in B- and C-LMP1 S6C Derived Tumors

The number of infiltrating mononuclear and polymorphonuclear leukocytes in B-LMP1 expressing S6CL5 tumors was increased compared to tumors derived from C-LMP1 expressing S6CLN7R (Fig. 3B,E). Quantitative image analysis of cryosections from two B-LMP1, three C-LMP1, one vector and two parental untransfected S6C derived tumors, stained with monoclonal anti-mouse CD45 antibody revealed a significant increase in CD45 immunoreactive cells in the B-LMP1 expressing S6CL5 derived tumors (Fig. 4, $P < 0.001$). The C-LMP1 expressing, the vector transfected and nontransfected tumors had significantly lower number of infiltrating leukocytes (Fig. 4).

ICAM-1 and B7 Expression in LMP1 Expressing S6C Cells

Figure 5 shows that neither B-LMP1 nor C-LMP1 significantly influenced the expression of ICAM-1 and B7-1 molecules in S6C cells. The mean fluorescence intensity (MFI) of ICAM-1 expression were 3.2, 5.32 and 2.43 for the vector, B- and C-LMP1 transfected S6C respectively. The MFI of CD80 expression for the above three cell lines were 7.85, 6 and 4.82. It was also found that MHC Class I and II expression was not altered in both transfectants (not shown).

DISCUSSION

Previous studies have shown that B-cell derived LMP1 was less transforming and strongly immunogenic when compared to NPC derived LMP1 [Hu et al., 1993; Trivedi et al., 1994]. In this report, it is demon-

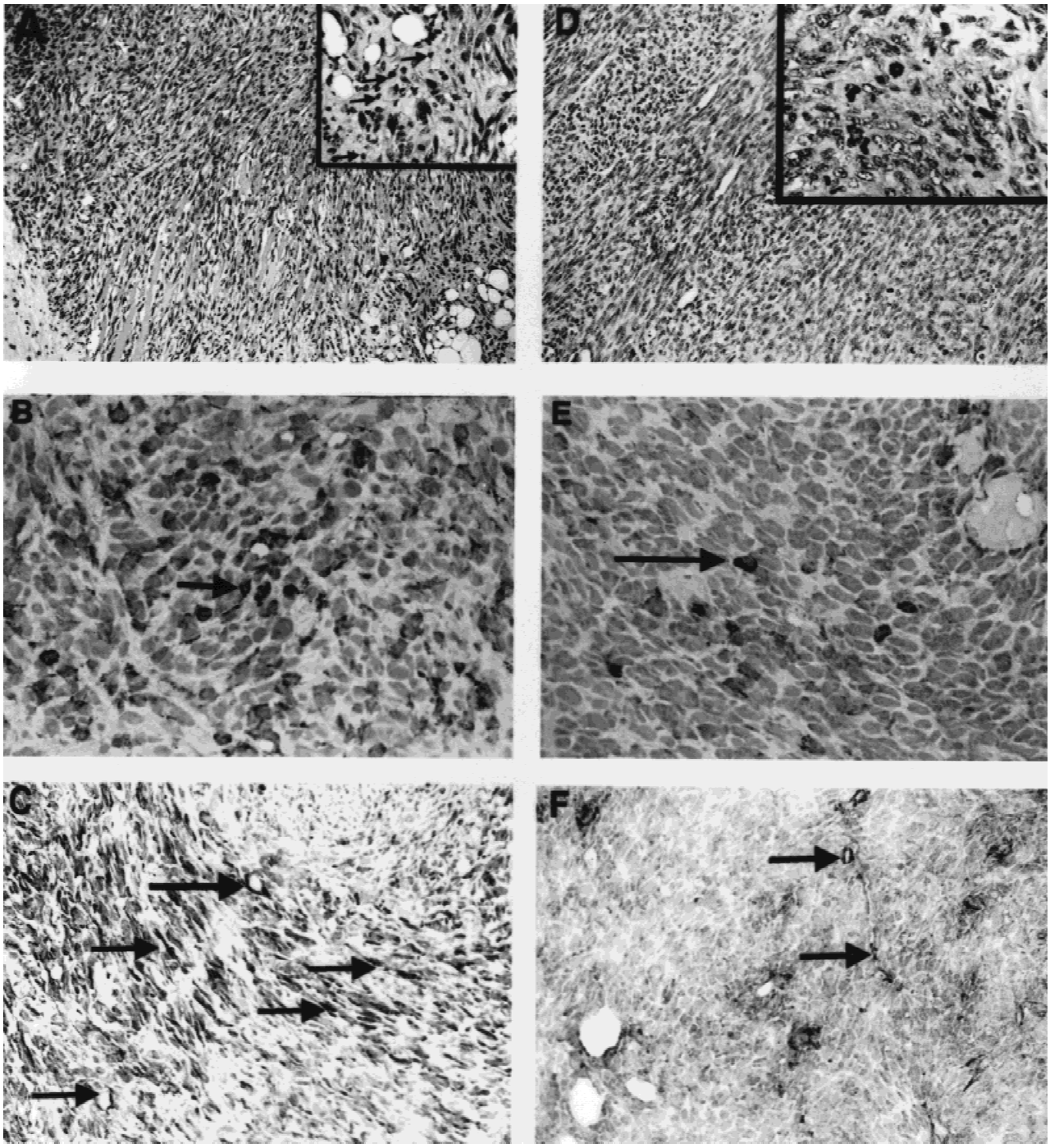

Fig. 3. Immunohistochemical analysis of B-LMP1 (A-C) and C-LMP1 (D-F) expressing S6C-derived tumors in non-immunized, immunocompetent, syngeneic mice. (A and D) Hematoxylin-eosin stained paraffin sections. The B-LMP1 expressing tumor (A: magnification ×100) shows infiltrative growth in loose subcutaneous tissue and between muscle bundles. The elongated tumor cells have a fibroblast-like morphology, with condensed chromatin and a low frequency of mitoses. An increased number of infiltrating mononuclear and polymorphonuclear leukocytes are seen (arrows in insert: magnification ×200). The C-LMP1 expressing tumor (D: magnification ×100) is more compact with a sarcomatous growth pattern with parallel bundles of spindle-shaped tumor cell. The chromatin is open and multiple nucleoli are seen as well as a high number of mitoses (insert: magnification ×250). (B and E) Cryostat sections immunohistochemically stained with a rat anti-mouse CD45 antibody; The arrows indicate examples of CD-45 positive cells. The B-LMP1 expressing tumor (B) reveals an increased number of immunostained leukocytes compared to the C-LMP1 expressing tumor (E); Magnification ×300. (C and F) Cryostat sections immunohistochemically stained with a monoclonal antibody to smooth muscle actin (SMA). The arrows indicate examples of SMA-stained vessels. The B-LMP1 expressing tumor (C) reveals an increased number of immunostained, often slit-like vessels in the stroma compared to the few vessels in the C-LMP1 expressing tumor (F); Magnification ×200.

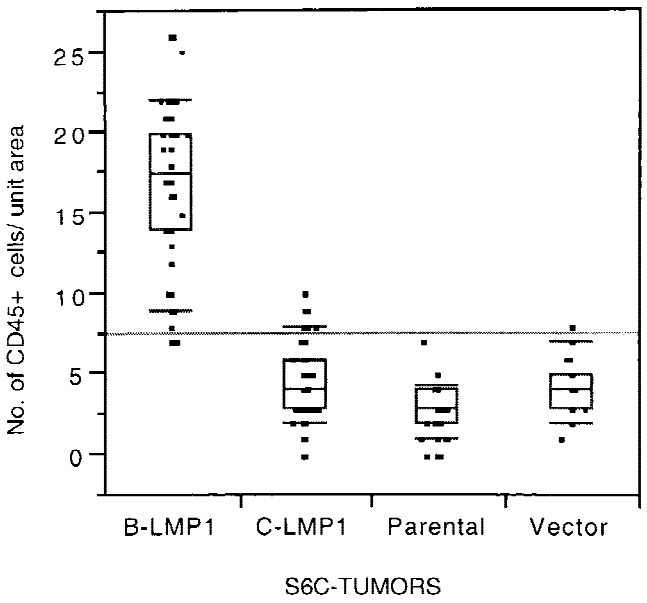

Fig. 4. Quantitative image analysis of cryosections of S6C tumors expressing B-LMP1, C-LMP1, vector and untransfected parenterals stained with anti mouse CD45 antibody. B-LMP1-1 represent tumors derived from two mice inoculated with S6CL5; C-LMP1 tumors are derived from three mice inoculated with S6CLN7R. The vector transfected S6C tumor derived from one mouse and parental S6C tumors are obtained from two mice inoculated with untransfected cells. The Quantile boxes: the lowest mark = 10%, the lower end of the box = 25%,then 50% and box top = 75% and the top mark = 90%.

strated that B-LMP1 expressing S6C tumors had an increased infiltration of CD45 positive leukocytes. In contrast, the tumor cells transfected with the non-immunogenic C-LMP1 isolate derived from CAO NPC did not show any significant difference in number of infiltrating leukocytes when compared to tumors derived from the untransfected or vector transfected S6C cells.

Tumor infiltrating leukocytes could represent anti-tumor immune reaction as well as the stromal elements that support tumor growth [Opdenakker and Van Damme, 1992]. The variable prognostic significance of tumor infiltrating leukocytes suggests that different types of interaction between tumor and host cells are possible [O'Sullivan and Lewis, 1994]. The infiltrating leukocytes can kill and establish an immune memory against the tumor cells. A subset of the infiltrating leukocytes can, however, support tumor growth and vascularization [Mantovani, 1992]. The final result of the tumor-host interaction, therefore, depends on the inhibitory and stimulatory signals produced both by tumor and infiltrating cells. As seen in Figure 3C, the increased vascular stroma on the one hand and an increased infiltration of leukocytes on the other hand, suggest that leukocyte infiltration could be facilitated directly by increased vascular stroma in B-LMP1 expressing S6C tumors resulting in necrosis and skin damage.

Anti-tumor immunity has been successfully generated with viral genes [Trivedi et al., 1991; Chen et al., 1991, 1992], immuno-accessory molecules and cytokine gene expression like B7, GM-CSF, IL-2, IL-4 and IL-12 [Chen et al., 1994; Sumimoto et al., 1997; Baskar et al., 1995; Connor et al., 1993; Stoppacciaro et al., 1997; Colombo et al., 1996]. The tumor rejection could also depend on inflammatory cytokines produced by the TILs [Litton et al., 1996]. In light of pleiotropic phenotypic changes brought about by LMP1 in a transfected cell [Zhang et al., 1995; Rowe et al., 1995], it is pertinent to ask whether the necrosis observed in B-LMP1 expressing tumors is due to upregulation of immuno-accessory proteins and/or inflammatory cytokines induced by LMP1 in the tumor or by the infiltrating leukocytes. We did not find significant differences in ICAM-1 and B7-1 expression in S6C expressing B- and C-LMP1 when compared to the untransfected parental cells. These findings indicate that B-LMP1 does not affect ICAM-1 expression in the cells of epithelial origin. Although, it remains to be determined whether there are differences in cytokine expression induced by B- and C-LMP1 S6C expressors. Furthermore, the B-LMP1 expressing S6C cells grew with severe skin damage in non-immunized, immunocompetent syngeneic mice but when tested in SCID mice, both B- and C-LMP1 tumors grew progressively without skin damage (Table I). This suggests that the skin damage and heavy necrosis in B-LMP1 expressing tumors might be the result of cytokines produced by the infiltrating leukocytes rather than those induced by LMP1 in the transfected cells; however, a co-operative effect of cytokines induced in B-LMP1 expressing S6C and those produced by the TILs in inducing necrosis can not be ruled out and this is presently under investigation.

How does the lack of TILs in C-LMP1 expressing tumors coincide with heavy lymphocytic infiltration [Zhong et al., 1991] seen in the NPCs? First, it is far from clear if such infiltrates in NPCs contain EBV specific responses. Moreover, recent studies show that lymphocytic infiltration and the NPC tumor cells produce cytokines that may even help the tumor growth. In other words, such infiltrations seem to have tumor promoting function rather than immunological suppression of it [Huang et al., 1999].

It should be noted that DNA sequences of Hodgkin's disease (HD) derived LMP1 isolates are similar to C-LMP1 [Knecht et al., 1993]. The lack of TILs in C-LMP1 expressing tumors is therefore of significance in view of the findings that EBV specific cytotoxicity is suppressed locally in HD [Frisan et al., 1995]. Additionally, in a single case reported so far on the EBV specific CTL responses derived from the infiltrating lymphocytes in HD, LMP2 or EBNA6 and not LMP1 were identified as targets in HLA A11 and B44 restriction [Dolcetti et al., 1995]. It will be interesting to test whether HD derived LMP1 would be functionally similar to CAO LMP1 in the present murine system.

It has been shown previously that LMP1 inhibits in vitro growth, agarose clonability and in vivo tumor growth of transfected BL41 cells in thymectomized and

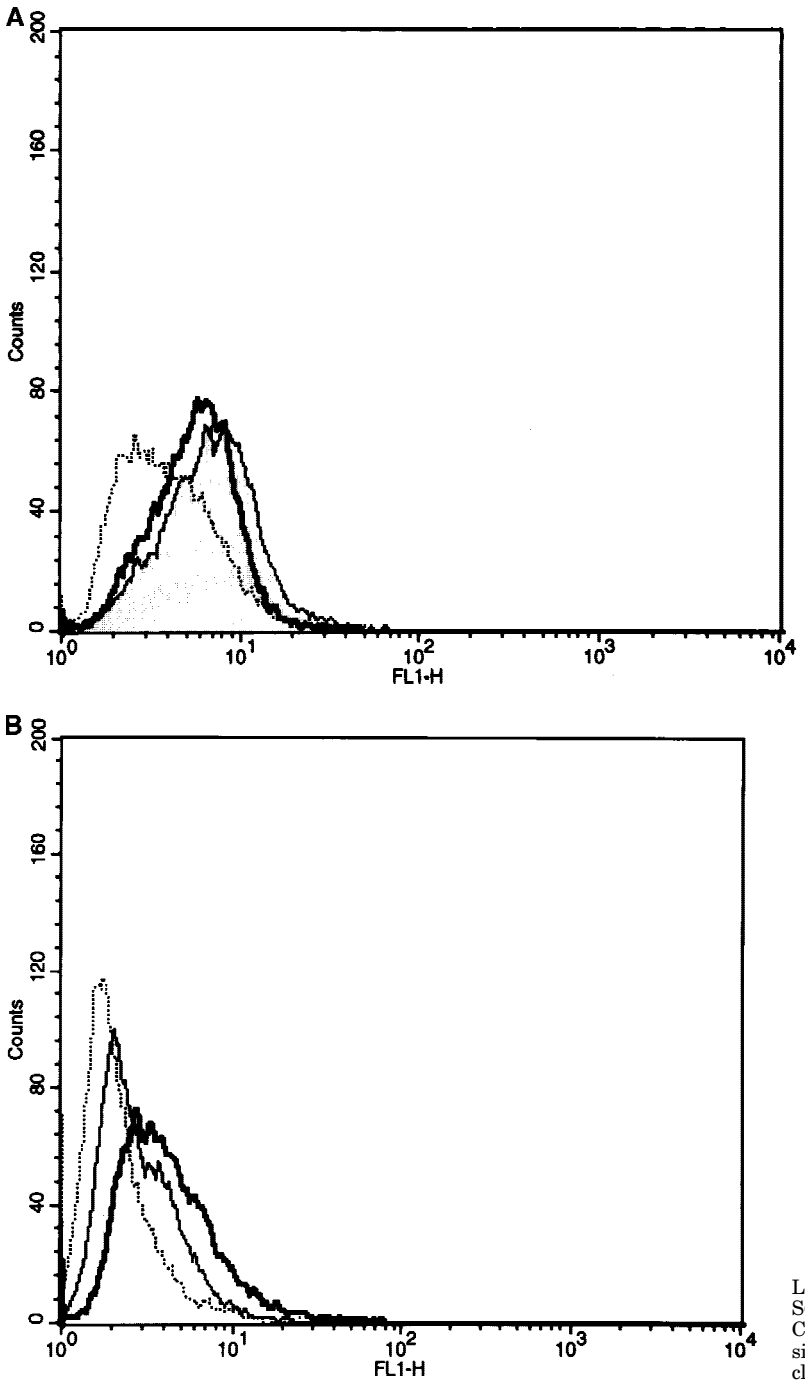

Fig. 5. (**A**) ICAM-1 expression in vector, B- and C-LMP1 transfected S6C. S6CV (....), S6CL5 (——) and S6CLN7R (▬)cells using FITC conjugated mouse anti-CD54 antibodies (Pharmingen). (**B**) B7-1 (CD80) expression in S6CV (....), S6CL5 (——), and S6CLN7R (▬) clones. One representative experiment out of five.

SCID mice [Cuomo et al., 1992; Floettmann et al., 1996]. More recently, Cherney et al. [1998] confirmed our findings using a nude mouse model and further demonstrated that LMP1 mediates tumor regression in T-cell independent manner. The present study adds three significant results to these data. Firstly, it shows that B cell derived LMP1 reduce tumor growth in non-immunized, syngeneic, immunocompetent mice. Secondly, B-LMP1 could recruit CD45+ leukocytes at the tumor site and thirdly, the CAO NPC derived LMP1 isolate is not capable of inducing CD45+ leukocyte infiltration in the tumors.

This report identified another functional difference between the two LMP1 isolates besides those earlier described [Hu et al., 1993; Trivedi et al., 1991]. Further studies will be required now for a precise phenotypic characterization of the tumor infiltrating leukocytes and to test whether isolates from LMP1 expressing and non-expressing NPCs would differ in such properties. Our studies underscore the potential of the B-cell de-

rived LMP1 in adoptive T cell transfer immunotherapy for EBV associated malignancies.

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